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# Phosphotyrosine profiling of human cerebrospinal fluid

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## Abstract

**Background:** Cerebrospinal fluid (CSF) is an important source of potential biomarkers that affect the brain. Biomarkers for neurodegenerative disorders are needed to assist in diagnosis, monitoring disease progression and evaluating efficacy of therapies. Recent studies have demonstrated the involvement of tyrosine kinases in neuronal cell death. Thus, neurodegeneration in the brain is related to altered tyrosine phosphorylation of proteins in the brain and identification of abnormally phosphorylated tyrosine peptides in CSF has the potential to ascertain candidate biomarkers for neurodegenerative disorders.

**Methods:** In this study, we used an antibody-based tyrosine phosphopeptide enrichment method coupled with high resolution Orbitrap Fusion Tribrid Lumos Fourier transform mass spectrometer to catalog tyrosine phosphorylated peptides from cerebrospinal fluid. The subset of identified tyrosine phosphorylated peptides was also validated using parallel reaction monitoring (PRM)-based targeted approach.

**Results:** To date, there are no published studies on global profiling of phosphotyrosine modifications of CSF proteins. We carried out phosphotyrosine profiling of CSF using an anti-phosphotyrosine antibody-based enrichment and analysis using high resolution Orbitrap Fusion Lumos mass spectrometer. We identified 111 phosphotyrosine peptides mapping to 66 proteins, which included 24 proteins which have not been identified in CSF previously. We then validated a set of 5 tyrosine phosphorylated peptides in an independent set of CSF samples from cognitively normal subjects, using a PRM-based targeted approach.

**Conclusions:** The findings from this deep phosphotyrosine profiling of CSF samples have the potential to identify novel disease-related phosphotyrosine-containing peptides in CSF.

**Keywords:** CSF, Phosphotyrosine, Proteome

## Background

Cerebrospinal fluid (CSF) is a colorless, clear liquid that surrounds the brain and spinal cord [1] and conventionally acts as a shock absorber in the central nervous system by maintaining electrolytes as well as acid–base balance [2]. It also plays a crucial role in neurodevelopment and waste clearance in addition. CSF is produced

from arterial blood by the choroid plexuses of the lateral and fourth ventricles. It also secretes nutrients, filters metabolites from the blood and eliminates waste products from the brain [3]. CSF regulates neuronal activity through neuropeptides and hormones [4]. As a proximal fluid, CSF reflects the health/disease state of the nervous system and is likely to have a higher concentration of protein biomarkers, which provide a direct readout of the state of the central nervous system [5]. Investigating the proteomic profile of this fluid can be beneficial in diagnosing various neurological disorders including Alzheimer's, Parkinson's diseases and multiple sclerosis.

Phosphorylation is one of the most commonly studied post-translational modifications (PTMs), and

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phosphorylation of proteins at specific sites are already used as biomarkers in neurological disorders (e.g.: phosphorylated tau in AD) [6–8]. Additionally, phosphorylation plays important roles in cell communication, signaling, aging, and cell adhesion [9, 10]. For example, phosphorylation of tyrosine residues by tyrosine kinases such as c-Abl has been reported to have a central role in neurodegeneration [11]. The biofluid phosphoproteome contains phosphorylated proteins either secreted from cells or leak from the intracellular content of damaged cells. Recent studies have shown that proteins may undergo phosphorylation in the extracellular compartment through the action of extracellular kinases [12, 13]. The discovery of novel secreted kinase VLK and Casein kinase, which are responsible for the phosphorylation of the major class of secreted proteins, has led to an increased interest in secreted phosphoproteins and their roles [14, 15]. Unfortunately, there are limited studies on direct analysis of phosphorylation in biofluids such as plasma phosphoproteins [16], CSF [17] and saliva [18]. Although several studies have been carried out on the proteomic profiling of CSF, limited studies were focused on its phosphoproteome [19–21]. Earlier targeted studies on neurological disorders focused on phosphorylation alteration on serine and threonine sites, including an increase in tau, synuclein phosphorylation in neurological disorders [22, 23]. Heegaard et al. in a previous study carried out phosphoproteomic profiling of cerebrospinal fluid using TiO<sub>2</sub> based enrichment. They identified 56 novel phosphorylation sites on 38 proteins [19]. In another study Nakamura et al. used titanium based and IMAC enrichment followed by use of EDTA for phosphopeptides analysis leading to identification of 123 phosphopeptides [20]. Molecularly imprinted polymers that have binding affinity sites for pS or pY were also used for enrichment of phosphopeptides from CSF. This led to identification of 47 phosphopeptides corresponding to 24 proteins [21]. All earlier mass spectrometry studies were focused on global phosphorylation of CSF.

Stoichiometrically, tyrosine phosphorylation is less abundant, and its detection is more difficult in global profiling although it plays an important role in regulation of neuronal maturation and synaptic plasticity [24]. Tyrosine phosphopeptides can be enriched using an antibody-based immunoprecipitation approach [25]. In an earlier study by Yuan X et al., phosphotyrosyl-proteins in CSF were detected using 2D gel electrophoresis. They identified four tyrosine phosphorylated proteins including kallikrein-6 precursor, complement C4 gamma-chain, gelsolin, and ceruloplasmin precursor. The major limitation of this approach was that the technology used was less sensitive and low throughput [26]. In this study, we carried out phosphotyrosine profiling to investigate the

tyrosine phosphorylation of proteins that are detectable in the CSF. Phosphotyrosine-containing peptides were enriched by an anti-phosphotyrosine antibody-based enrichment method followed by high-resolution mass spectrometry.

## Methods

### Materials

Anti-phosphotyrosine rabbit monoclonal antibody (P-Tyr-1000) beads were obtained from Cell Signaling Technology (Danvers, MA). TPCK-treated trypsin was obtained from Worthington Biochemical Corp. (Lakewood, NJ). All other reagents used in this study were from Fisher Scientific (Pittsburgh, PA).

### Lumbar CSF samples

The sample group consisted of subjects with normal pressure hydrocephalus (NPH) and cognitively normal individuals seen as part of the BIOCARD study [27]. The participants underwent a lumbar puncture in the fasted state in the morning and the CSF was collected in polypropylene vials and stored at –80 °C. NPH CSF was collected via an indwelling lumbar catheter and in cognitively normal individuals by lumbar puncture. We have collected CSF samples from 3 NPH and 8 cognitively normal individuals. All individuals selected for inclusion within the study provided informed consent prior to sample collection.

### Protein concentration and in-solution trypsin digestion of proteins from CSF

CSF samples were adjusted to 20 mM HEPES, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate. CSF was concentrated 10 times by volume using 3 kDa cutoff spin columns (Microcon Ultracel YM-3, Millipore). The concentration of protein from these samples was measured using the BCA protein estimation method. We used protein from 100 ml CSF from all three samples for the in-solution digestion and the amount of the proteins present in the samples was respectively 49, 51 and 52 mg. For reduction, dithiothreitol (Sigma) was added to a final concentration of 10 mM and the mixture was incubated at 56 °C for 45 min. For alkylation, the mixture was cooled to room temperature and iodoacetamide was added to a final concentration of 20 mM and incubated at room temperature for 45 min in the dark. CSF samples were subjected to digestion with TPCK treated trypsin with 1:50 trypsin to CSF protein ratio (Worthington Biochemical Corp, Lakewood, NJ) for 12–16 h at room temperature. Protein digests were acidified by 1% trifluoroacetic acid (TFA), centrifuged at 12,000×g for 5 min and supernatant desalted using

C<sub>18</sub> Sep-Pak cartridge (Waters, Cat#WAT051910) and lyophilized.

#### Immunoaffinity purification of tyrosine phosphopeptides

The lyophilized peptide mixtures were dissolved in IAP buffer containing 50 mM MOPS pH 7.2, 10 mM sodium phosphate and 50 mM NaCl. Prior to phosphotyrosine enrichment, the P-Tyr-1000 beads (Cell Signaling Technology, Danvers, MA) were washed twice with IAP buffer at 4 °C. The peptide mixtures were then incubated with P-Tyr-1000 beads for 30 min with gentle rotation. To remove non-specifically bound peptides, the beads were washed thrice with ice cold IAP buffer and twice with ice cold water. Elution of enriched peptides from beads was carried out at room temperature using 0.15% TFA. This step was repeated twice and followed by cleanup of the samples using C<sub>18</sub> Stage tips.

#### LC-MS/MS analysis of enriched peptides

The enriched phosphotyrosine-containing peptides were analyzed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Electron, Bremen, Germany) interfaced with EASY-nLC II nanoflow liquid chromatography system equipped with an EASY-Spray ion source. (Thermo Scientific, Odense, Denmark). Peptide digests were reconstituted in 0.1% formic acid and loaded onto trap column (Thermo Scientific™ Acclaim™ PepMap™ 100 C18 LC) at a flow rate of 3 µl/min. Peptides were separated on an analytical column (EASY-Spray™ LC) at a flow rate of 300 nl/min using a step gradient of 5–18% solvent B (0.1% formic acid in 95% acetonitrile) for first 110 min and 18–30% solvent B for 110–160 min. The total run time was set to 180 min. The mass spectrometer was operated in a data-dependent acquisition mode. A survey full scan MS (from *m/z* 350–1500) was acquired in the Orbitrap with a resolution of 120,000 at 400 *m/z*. The AGC target for MS1 was set as  $1 \times 10^6$  and ion filling time set 60 ms. The most intense ions with charge state  $\geq 2$  were isolated in 3 s cycle and fragmented using HCD fragmentation with 32% normalized collision energy and detected at a mass resolution of 30,000 at 200 *m/z*. The AGC target for MS/MS was set as  $5 \times 10^4$  and ion filling time set 200 ms dynamic exclusion was set for 30 s with a 10 ppm mass window.

For the PRM analysis, the enriched phosphotyrosine containing peptides were analyzed on an Orbitrap Fusion Tribrid mass spectrometer interfaced with EASY-nLC II nanoflow liquid chromatography system. Peptide digests were reconstituted in 0.1% formic acid and loaded onto a trap column at a flow rate of 3 µl/min and resolved on analytical column. The mass spectrometer was operated in data-independent acquisition PRM mode. A survey full scan MS (from *m/z* 350–1700) was acquired in the

Orbitrap at a resolution of 120,000 at 400 *m/z*. A targeted list of precursor ions with charge state  $\geq 2$  were isolated and fragmented using HCD fragmentation with 32% normalized collision and detected at a mass resolution of 30,000 at 400 *m/z*. The data were subsequently analyzed using Skyline [28].

#### Data analysis

The MS/MS database searches were carried out using SEQUEST search algorithms against RefSeq human protein database using Proteome Discoverer 2.1 (Thermo Fisher Scientific, Bremen, Germany). The workflow included spectrum selector, SEQUEST search nodes, peptide validator and phosphoRS nodes. Oxidation of methionine, phosphorylation at serine, threonine and tyrosine (+79.966 Da) were set as variable modifications and carbamidomethylation of cysteine was set as a fixed modification. MS and MS/MS mass tolerances were set to 10 ppm and 0.05 Da, respectively. Trypsin was specified as protease and a maximum of one missed cleavage was allowed. Target-decoy database searches used for calculation of false discovery rate (FDR) and for peptide identification FDR was set at 1%. The probability of the phosphorylation site was calculated using phosphoRS 3.1 node in the Proteome Discoverer. Phosphopeptides with >75% localization probability was considered for further analysis.

#### Availability of data

The mass spectrometry derived data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with the dataset identifier PXD009152.

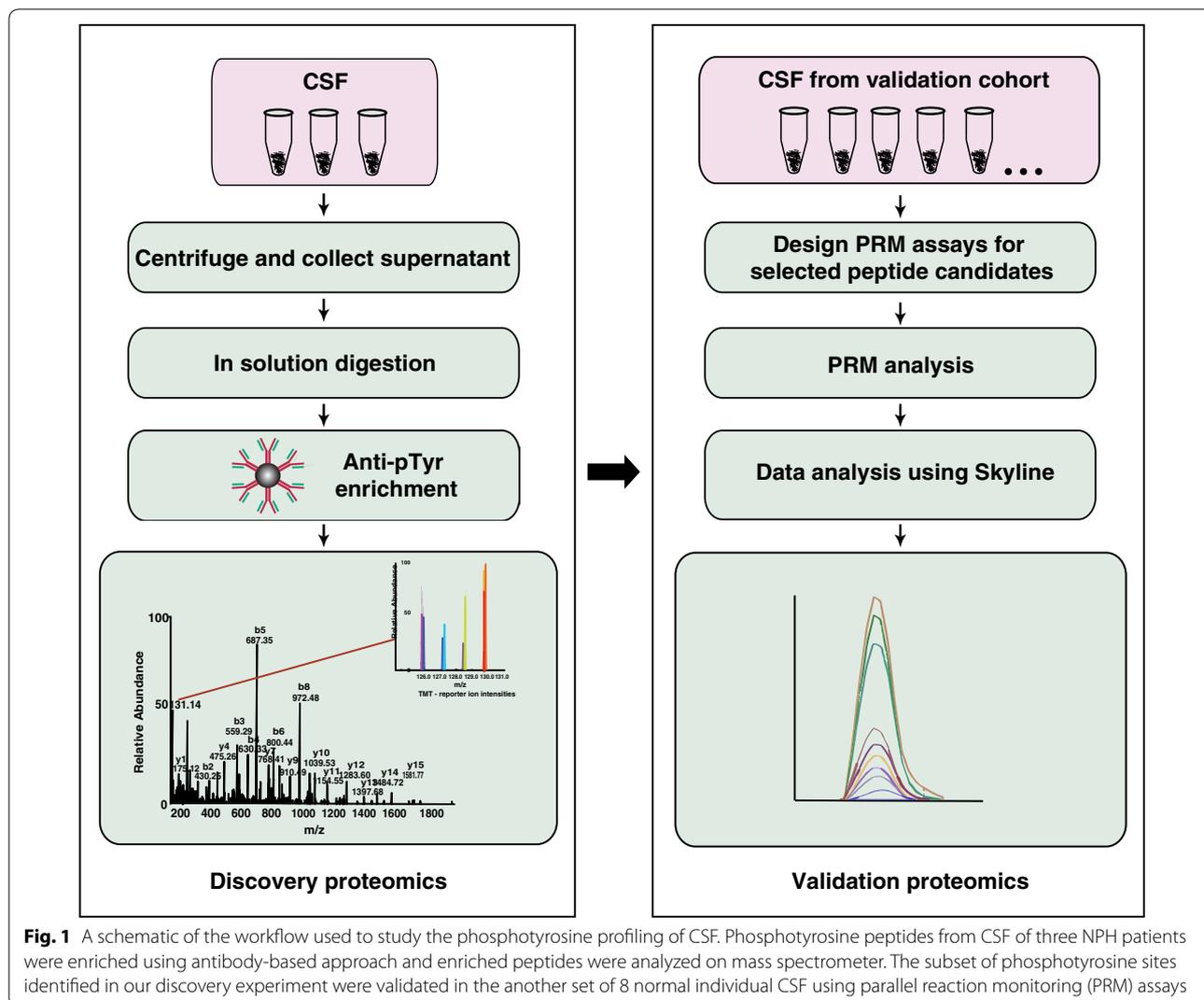
#### Bioinformatics analysis

Molecular function and localization of phosphoproteins was obtained from the Human Protein Reference Database (HPRD) [29]. The involvement of these phosphoproteins in biological processes was also obtained from HPRD.

## Results

#### LC-MS/MS analysis of tyrosine phosphorylated peptides in CSF

The CSF proteins were digested with trypsin, phosphotyrosine peptides were enriched, and LC-MS/MS analysis was conducted as described in Methods [25, 30, 31] (Fig. 1). We identified 111 phosphotyrosine peptides mapping to 66 proteins (Additional file 1: Table S1) of which 38 were identified from at least two samples (Fig. 2a). These identified phosphotyrosine peptides are corresponding to 66 proteins (Additional file 2: Table S2). We have used tyrosine phosphorylated peptide

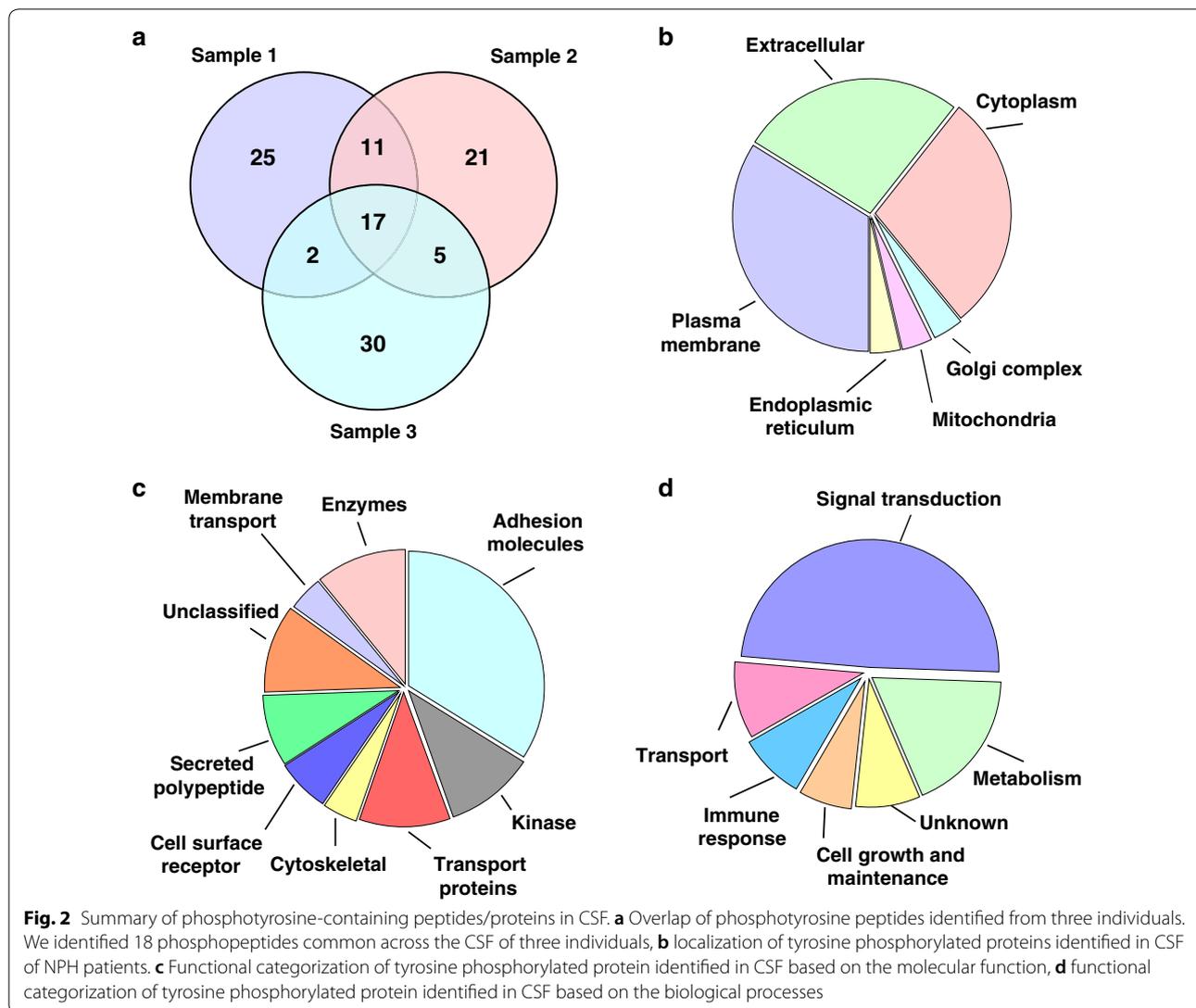


immunoaffinity enrichment-based technique along with high-resolution mass spectrometry on an Orbitrap Fusion Lumos Tribrid mass spectrometer—the latest generation mass spectrometer for deep characterization of phosphotyrosine peptides in CSF.

**Classification of tyrosine phosphorylated CSF proteins using gene ontology**

To obtain deeper biological insights into the identified CSF tyrosine phosphorylated proteins, we categorized them based on their subcellular localization, molecular function and biological process (Fig. 2b–d). The secretory proteins were mostly hormones, cytokines and proteases that possess important regulatory functions and are released for cell to cell interaction and communication. Of the 66 proteins identified, 33 proteins have signal peptides and 13 have transmembrane domains. This analysis reveals that the majority of tyrosine phosphorylated

proteins in CSF are destined towards the secretory pathway. Our analysis showed that a majority of the tyrosine phosphorylated proteins were localized to the plasma membrane (34%), followed by extracellular matrix (26%), cytosol (26%) and endoplasmic reticulum (4%) (Fig. 2b). Most of the proteins were found to be involved in cell adhesion activity, transport activity or kinase activity (Fig. 2c). A majority of the proteins identified were cell adhesion proteins, which included CHL1, SPP1 and NRXN3. The CHL1 cell adhesion molecule L1 like is a neural cell adhesion molecules and it is significantly associated with schizophrenia [32, 33]. SPP1 (secreted phosphoprotein 1) and NRXN3 (neurexin 3) are already known to be associated with Alzheimer’s disease [34, 35]. The other major categories of proteins identified were kinases (15%), cytoskeletal proteins (14%), membrane and cell junction proteins (13%) and the transport/ carrier proteins. The identified CSF proteins were also



categorized based on their biological processes. Most of these proteins were found to be involved in cell communication and signal transduction, followed by transport, cell growth and energy pathways as shown in Fig. 2d.

### Targeted analysis of a subset of tyrosine phosphorylated peptides in CSF

The results from the discovery experiments were subsequently validated in CSF of cognitively normal individuals (N=8) using a targeted parallel reaction monitoring (PRM) approach. We selected a set of tyrosine phosphorylated peptides based on their abundance and biological role for analysis in PRM mode. The tyrosine phosphorylated peptides identified in normal individual CSF using PRM mode includes proteins DBI (diazepam binding inhibitor), acyl-CoA binding protein, B4GAT1 (beta-1,

3-N-acetylglucosaminyltransferase), CD59, transferrin and Thy-1 cell surface antigen. The list of the tyrosine phosphorylated peptides and its corresponding proteins are shown in Table 1. The relative abundance of

**Table 1** A list of proteins that are validated using PRM

Gene symbol	Protein	Peptide sequence	Site
<i>DBI</i>	Diazepam binding inhibitor, acyl-CoA binding protein	TKPSDEEMLFYGHYK	Y90
<i>TF</i>	Transferrin	EGYYGTGAFR	Y536
<i>B4GAT1</i>	Beta-1, 4-glucuronyltransferase 1	YEAAVDPDR	Y163
<i>CD59</i>	CD59 glycoprotein prepro-protein	ENELTYYCCK	Y86
<i>THY-1</i>	Thy-1 cell surface antigen	VLVLSAFTSK	Y90

the each of the phosphopeptides in the different samples is shown in Additional file 3: Figure S1.

## Discussion

### Phosphotyrosine profiling of cerebrospinal fluid

Since the alterations in tyrosine phosphorylation of proteins in the brain can be reflected in the CSF, such proteins can be excellent candidates as biomarkers for neurodegenerative diseases such as AD and PD. In spite of this, there are no reports that describe phosphotyrosine profiling of proteins in CSF. Proteins can be phosphorylated on serine, threonine and tyrosine. Of these, the phosphorylation on tyrosine residues is much less abundant than either serine or threonine residues. Owing to this, the identification of phosphotyrosine modifications is mostly conducted after enriching phosphotyrosine-containing peptides using anti-phosphotyrosine antibodies from large amounts of proteins (> 20 mg). Because the protein concentration of CSF is only ~0.15 to 0.6 mg/ml, large volumes of CSF are required for such an analysis. To overcome this limitation, we used three sets of CSF samples (100 ml each generating ~50 mg protein in each instance) obtained from subjects with NPH and processed them independently. In this study, we employed antibody-based enrichment with Orbitrap Fusion Lumos Tribrid mass spectrometer—the latest generation mass spectrometer for identification of tyrosine phosphorylation in the cerebrospinal fluid. We identified 111 phosphotyrosine peptides corresponds to 66 proteins.

### Proteins uniquely identified in this study

To identify proteins which have not been previously reported in the CSF, we compared our data with proteins cataloged in the CSF-PR database. CSF-PR is an online repository of mass spectrometry-based proteomics experiments on human cerebrospinal fluid [36]. We identified 24 proteins that have not been previously detected in cerebrospinal fluid. A partial list of uniquely identified proteins in this study is provided in Table 2. This list includes proteins CWF19 like 1, cell cycle control (CWF19L1), inositol polyphosphate-5-phosphatase D (INPP5D), beta-1, 4-glucuronyltransferase 1 (B4GAT1), protein kinase C delta (PRKCD), NCK adaptor protein 2 (NCK2), glucose-6-phosphate dehydrogenase (G6PD).

Mutations in the CWF19 gene, which encodes CWF19 protein family have been associated with ataxia and mild mental retardation [37]. CWF19 has a strong association with late-onset Alzheimer's disease [38]. Our study identified phosphorylation of CWF19 at Y192 and T186 as it was located on the same phosphopeptide. Phosphorylation of these sites has not been reported previously. The representative MS/MS for the doubly phosphorylated peptide is shown in Fig. 3a. Glucose-6-phosphate dehydrogenase is a cytosolic enzyme and its main function is to produce NADPH, an important electron donor. Activity of G6PD is increased in the inferior temporal cortex of Alzheimer individuals [39]. G6PD plays an important role in compensating oxidative stress produced in Alzheimer's disease [40]. In another study, G6PD enzyme activity in serum of AD and control subjects was measured. G6PD activity is two times higher in AD patients as compared to controls [41]. We identified G6PD phosphorylation at

**Table 2** A list of proteins identified that are unique in this study

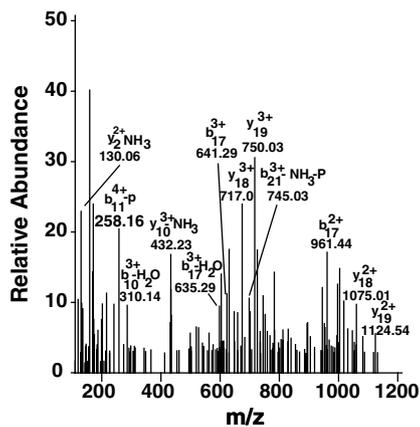
Gene symbol	Protein	Peptide sequence	Site
<i>CWF19L1</i>	CWF19 like 1, cell cycle control	CGSALVSSLATGLKPRYHFAALEK	Y192
<i>INPP5D</i>	Inositol polyphosphate-5-phosphatase D	EKLYDFVK	Y865
<i>B4GAT1</i>	Beta-1, 4-glucuronyltransferase 1	YEAAVDPDR	Y163
<i>PTPN11</i>	Tyrosine-protein phosphatase non-receptor type 11	IQNTGDYYDLYGGEK	Y584
<i>G6PD</i>	Glucose-6-phosphate dehydrogenase	VQPNEAVYTK	Y432
<i>FGR</i>	FGR proto-oncogene	LIKDDEYNPCQGSK	Y412
<i>PXN</i>	Paxillin	VGEEEHVYSFPNK	Y124
<i>CALM2</i>	Calmodulin 2	DGNGYISAAELR	Y148

(See figure on next page.)

**Fig. 3** MS/MS spectra for the phosphotyrosine-containing peptides/proteins uniquely identified in ours study. **a** MS/MS spectra for doubly phosphorylated peptide identified from CWF19, **b** representative MS/MS spectra for phosphorylated peptide identified from G6PD, **c** representative MS/MS spectra for phosphorylated peptide identified from PTPN6 and **d** representative MS/MS spectra for phosphorylated peptide identified from PXN

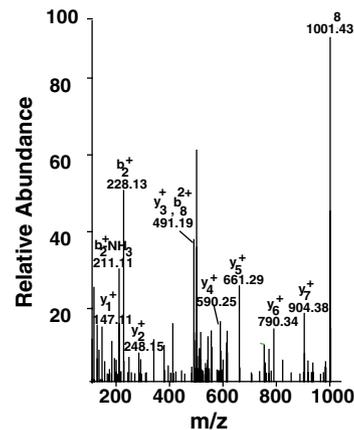
**a** CWF19L1  
CGSALVSSLATGLKPR<sup>Y</sup>HFAALEK

b <sup>+</sup>	b <sup>2+</sup>	b <sup>3+</sup>	b <sup>4+</sup>	Seq.	y <sup>+</sup>	y <sup>2+</sup>	y <sup>3+</sup>	y <sup>4+</sup>
81.02280	94.35080	41.01464	31.01341	C-Cysteine				
189.53333	71.35798	55.27030	44.41770	G	2576.26737	1288.63732	859.42731	644.82230
153.04925	102.36986	77.02031	61.02410	S	2513.24930	1256.12059	840.42075	630.56033
186.96790	126.64769	94.78789	76.03750	A	2432.21387	1216.10596	811.40948	608.68893
245.10994	143.14238	123.05681	96.64834	L	2361.17676	1181.03002	787.73944	591.64905
294.64414	186.18178	147.62871	118.46202	V	2280.00710	1124.94999	750.02075	562.73963
236.16016	225.77956	160.93072	126.06643	S	2149.02420	1075.01579	717.01295	530.01193
381.67617	254.78854	191.34172	153.27483	S	2061.98225	1031.49877	688.00217	516.25292
438.21820	282.48123	214.01214	175.09160	L	1974.96822	987.38078	658.99199	484.45551
473.73676	316.16027	237.37202	190.09007	A	1881.87616	931.44712	621.25691	466.22490
564.24376	376.49827	280.62952	226.30937	T-Phospho	1790.83905	895.82316	597.61787	448.48252
552.75490	385.05442	286.89889	227.78216	D	1689.82044	806.41616	512.27986	403.21122
649.29893	433.20011	325.15190	260.32298	L	1552.80768	776.90543	518.27271	388.95626
713.34481	475.09843	357.17954	285.94797	K	1423.71951	720.30329	488.57892	360.68534
781.87828	500.24826	381.43663	305.25262	F	1211.62495	606.21991	412.87930	328.64193
839.32095	560.26306	420.46411	326.57274	R	1214.57178	607.70953	405.52878	304.26840
901.42676	641.26294	481.22753	365.11660	Y-Phospho	1098.47887	529.78988	351.49588	265.27313
1029.96523	686.97925	515.48825	412.59046	H	815.44101	408.22415	272.46519	204.61571
1163.49944	736.00205	552.25236	442.00414	F	678.38210	339.69489	226.79889	170.26098
1120.01940	789.89109	570.01264	456.21756	A	521.31380	266.15448	177.77688	133.56888
1174.53695	783.26013	587.77192	470.41899	A	460.27857	236.64193	154.09794	115.62460
1221.07097	821.05462	616.04283	493.02980	L	303.22946	155.12297	130.41890	90.90532
1295.05888	884.49001	648.37058	518.84422	E	276.19540	138.90134	92.72252	69.79471
				K	147.11280	74.06004	49.70932	37.52386



**b** G6PD  
VQPNEAV<sup>Y</sup>TK

b <sup>+</sup>	b <sup>2+</sup>	Seq.	y <sup>+</sup>	y <sup>2+</sup>
100.07569	50.54148	V		
228.13427	114.57077	Q	1129.49253	565.24990
325.18703	163.09715	P	1001.43395	501.22062
439.22996	220.11862	N	904.38119	452.69423
568.27255	284.63991	E	790.33826	395.67277
639.30967	320.15847	A	661.29567	331.15147
738.37808	369.63268	V	590.25856	295.63292
981.40774	491.20751	Y-Phospho	491.19014	246.09871
1082.45542	541.73135	T	248.16048	124.58388
		K	147.11280	74.06004



Y401 in CSF. The representative MS/MS spectra is shown in the Fig. 3b.

PTPN6 belongs to a member of the protein tyrosine phosphatase (PTP) family. It involved in the cell differentiation, cell growth, differentiation, mitotic cycle, and oncogenic transformation [42]. PTPN6 expression was increased in glioma and its expression correlating with poor survival. Its expression controlled epigenetically and impacts the response to chemotherapy [43]. In our analysis, we detected phosphorylation at Y 584. The MS/MS spectrum for this peptide is shown in Fig. 3c. Paxillin (PXN) is involved in actin-membrane attachment at sites of cell adhesion to the extracellular matrix. It is an adapter protein which plays an important scaffolding role at focal adhesions by employing structural and signaling molecules involved in cell movement and migration, when phosphorylated on specific Tyr and Ser residues [44]. We have detected phosphorylated paxillin in CSF. The MS/MS of the phosphopeptide is shown in the Fig. 3d. These newly identified CSF proteins will help to increase knowledge of extracellular signaling and understanding pathology of neurological disorders.

#### Targeted analysis of tyrosine phosphorylated CSF proteins

The results from the discovery experiments were subsequently validated in CSF of cognitively normal individuals using a targeted parallel reaction monitoring (PRM) approach. This tyrosine phosphorylated peptides corresponds to the proteins DBI (diazepam binding inhibitor), acyl-CoA binding protein, B4GAT1 (beta-1, 3-N-acetylglucosaminyltransferase), CD59, transferrin and Thy-1 cell surface antigen. Validation of these tyrosine phosphorylated peptides in 8 CSF samples from cognitively normal healthy controls is a preliminary finding that needs to be replicated in a larger cohort. Unlike other biofluids, CSF is difficult to obtain, especially CSF from cognitively normal healthy controls. Although the sample size for the validation is small, these findings are quite promising.

DBI (diazepam binding inhibitor) is a polypeptide that was initially identified from rat brains [45]. This protein is involved in lipid metabolism and the displacement of beta-carbolines and benzodiazepines, which regulate signal transduction at type-a gamma-aminobutyric acid receptor located in brain synapses. Multiple missense mutations have been reported in the DBI gene in schizophrenia [46]. It regulates steroidogenesis in mitochondria and also glucose-stimulated insulin secretion [47]. DBI gene polymorphisms are also associated with anxiety disorders [48]. As DBI can be a potential candidate gene for psychiatric phenotypes including anxiety, mood, and psychotic disorders, its concentration has been investigated in the plasma of epileptic patients and found to be

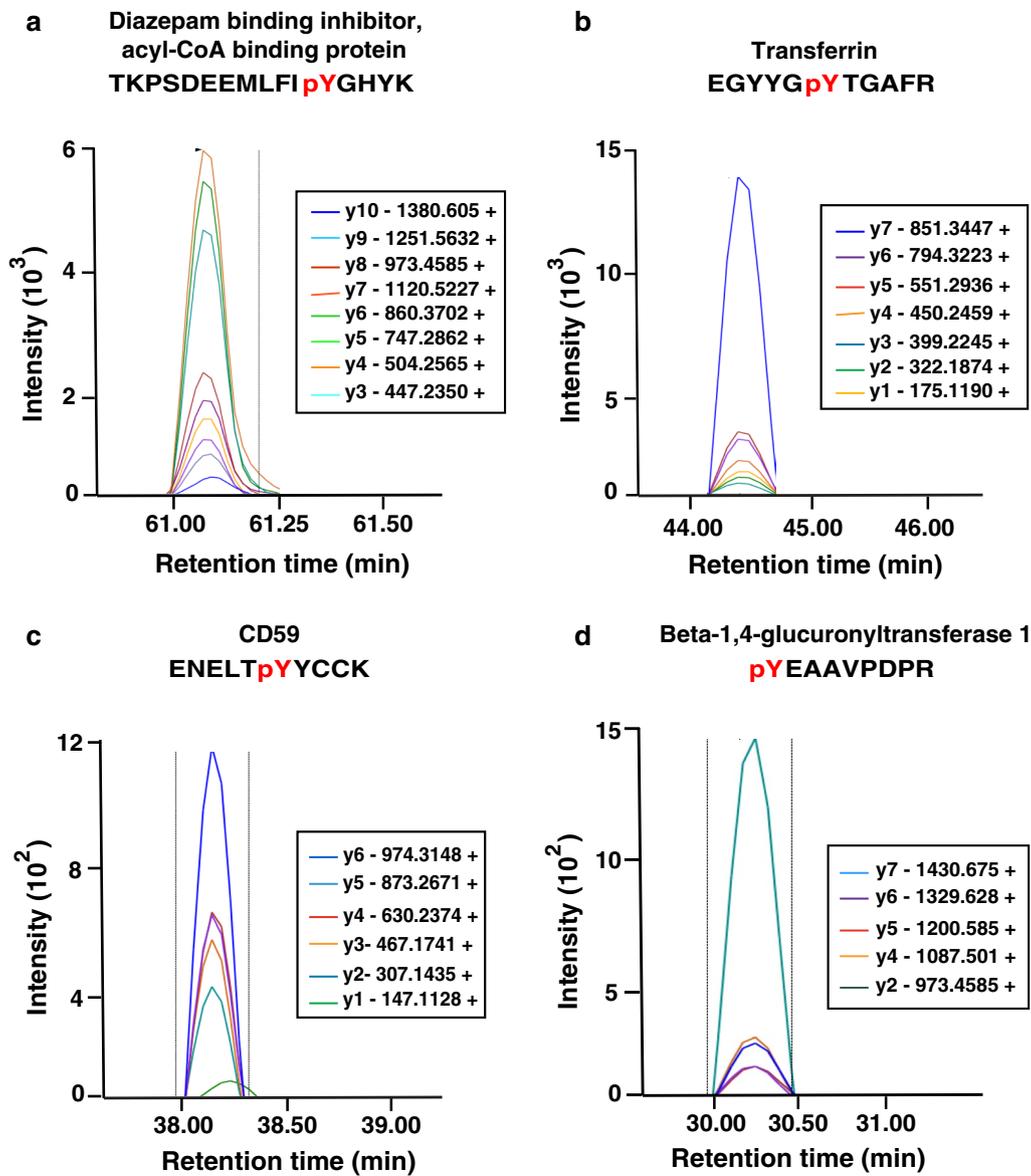
elevated [49]. The transitions for the peptide corresponding to phosphorylated DBI are shown in Fig. 4a.

Transferrin plays important roles in iron transport from the intestine, reticuloendothelial system, and liver parenchymal cells to all cells in the body for proliferation. It also plays important roles in the removal of allergens from the serum. Several mass-spectrometry based method were established for the detection of glycoforms for diagnosis of congenital disorders [50]. Mutations in transferrin lead to an increased risk of Alzheimer's disease [51]. The N-glycan proteoform of CSF transferrin is a potential biomarker for normal pressure hydrocephalus (iNPH) [52]. Transferrin has recently been shown to be a potent in vitro inhibitor of self-association of A $\beta$  [53]. The transitions for the peptide corresponding to phosphorylated transferrin are shown in Fig. 4b.

CD 59 is a surface glycoprotein that controls complement-mediated cell lysis, which has been identified previously in plasma, urine, saliva and CSF [54]. It has been reported that the concentration of CD 59 is elevated in multiple sclerosis patients [55]. We have identified phosphorylation of CD 59 at Y87. The transitions for the peptide corresponding to phosphorylated CD 59 are shown in Fig. 4c. Thy-1 antigen is a cell surface glycoprotein, which is the most abundant glycoprotein in mammalian neurons [56, 57]. It has also been reported as a cancer stem cell marker in high grade gliomas [58]. In the nervous system, Thy-1 plays an important role in cell communication [59]. We have detected tyrosine phosphorylated sites on Thy-1 in CSF samples using the PRM approach. B4GAT1 is a member of the beta-1,3-N-acetylglucosaminyltransferase family and it is a transmembrane protein having enzymatic activity. A truncating mutation in B4GAT1 is known to causes severe Walker-Warburg syndrome, a congenital muscular dystrophy [60]. Dystroglycan organizes basement of cell membrane by interacting ligands in the extracellular matrix. BGAT1 play important role in activation of dystroglycan [61]. The transitions for the peptide corresponding to phosphorylated Beta-1,4-glucuronyltransferase 1 are shown in Fig. 4d. The PRM analysis of normal CSF samples confirms that these tyrosine phosphorylated peptides are easily detected in individual normal CSF samples using the PRM approach.

#### Conclusions

In this study, we have investigated tyrosine phosphorylation in cerebrospinal fluid and identified several novel phosphosites. The study represents the first deep phosphotyrosine proteome profiling of cerebrospinal fluid. Further work is necessary in larger cohorts comparing cognitively normal and diseased subjects to determine if any of these phosphoproteins play a role in



**Fig. 4** Validation of tyrosine phosphorylated peptides in control CSF samples by parallel reaction monitoring. **a** Diazepam binding inhibitor, acyl-CoA binding protein (DBI), **b** transferrin (TF) and **c** CD 59 and **d** beta-1,4-glucuronyltransferase 1(B4GAT1)

pathophysiology or can serve as biomarkers in neurological disorders.

**Additional files**

**Additional file 1: Table S1.** A summary of tyrosine phosphopeptides identified in CSF.

**Additional file 2: Table S2.** A summary of tyrosine phosphorylated proteins identified in CSF.

**Additional file 3: Figure S1.** A relative abundance of the tyrosine phosphorylated peptides in the CSF samples.

**Authors' contributions**

GS, AM and AP conceived the idea and planned the study. GS carried out sample preparation, mass spectrometry and bioinformatics analysis. GS wrote the manuscript. GS and AM prepared figures and tables. AP, CNA, SR, AM and MA provided critical inputs and revised the manuscript. All authors read and approved the final manuscript.

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#### Competing interests

The authors declare that they have no competing interests.

#### Availability of data and materials

Not applicable.

#### Consent for publication

All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

All individuals selected for inclusion within the study provided informed consented prior to sample collection.

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